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Dissertação

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Resumo

Cooperação é geralmente definida como qualquer interacção entre indivíduos em que os custos de investimento nessa parceria são suplantados pelos seus benefícios. Quando as interacções se desenrolam entre indivíduos de espécies distintas, são referidas como mutualismos. Neles se incluem os mutualismos de limpeza. Os mutualismos de limpeza envolvem duas partes: um limpador, de pequenas dimensões e com uma coloração conspícua, e um cliente, geralmente de maiores dimensões. Os organismos que estabelecem este tipo de cooperação acabam por desenvolver adaptações ao nível morfológico e comportamental que facilitam a interacção. O bodião limpador *Labroides dimidiatus*, juntamente com um restrito número de espécies das famílias *Labridae* e *Gobiidae*, apresenta o mais alto nível de especialização em comportamentos de limpeza, sendo classificado como limpador obrigatório, ou seja, a sua alimentação baseia-se exclusivamente no que consegue remover dos seus clientes. Os limpadores encontram-se em territórios específicos, designados por estações de limpeza, e vivem num sistema de harém (espécie poligínica e protogínica).

Numa interacção de limpeza, os limpadores removem da superfície dos seus clientes ectoparasitas e tecido infectado, ferido ou morto. A interacção é geralmente iniciada pelos clientes, que adoptam uma posição específica ficando imóveis e abrindo a boca, opérculos e barbatanas peitorais, mostrando assim que desejam ser limpos. Por usa vez, os limpadores adoptam um comportamento específico de dança, nadando para cima e para baixo. Esta dança não só chama a atenção dos seus clientes, como também serve como estratégia de pré-conflito, pois reduz a agressividade dos seus clientes. Outra estratégia pré-conflito utilizada pelos limpadores é a estimulação táctil, durante a qual, usando as barbatanas pélvicas e peitorais, "massajam" o corpo do cliente durante a inspecção. As massagens não só exercem um efeito anti-*stress* sobre o cliente, como também aumentam a sua *fitness*. Por causa disso, os limpadores manipulam os seus clientes a ficarem mais tempo na estação de limpeza,

mesmo depois de um acto desonesto. Os limpadores são desonestos quando removem muco e escamas dos clientes, que os limpadores preferem, mas que é prejudicial aos clientes pois requerem alocação de energia para reposição dos mesmos. Por ser a sua preferência, os limpadores cativam clientes de maior valor nutricional, isto é, com maior carga parasitária e/ou muco de melhor qualidade, a aproximarem-se para serem inspecionados. Para tal, os limpadores dão massagens e limpam clientes de menor valor, mostrando assim uma boa qualidade de serviço aos clientes de maior valor, que são cativados a recorrerem a esses limpadores para uma qualidade de serviço idêntica. No entanto, quando se aproximam para serem inspeccionados, os limpadores são desonestos e mordem o muco destes clientes. Para se certificarem que não voltam a ser enganados, os clientes punem os limpadores de formas diferentes. Os clientes com acesso a mais estações de limpeza fogem imediatamente ao comportamento desonesto, escolhendo outra estação de limpeza para a sua próxima inspecção, enquanto que os clientes residentes perseguem activamente o limpador desonesto.

Para estas interacções funcionarem correctamente, o limpador não pode ser sempre desonesto, mesmo que a desonestidade lhe traga benefícios imediatos maiores. A desonestidade faz com que os clientes não queiram voltar à mesma estação, e sem um bom fluxo de clientes, os limpadores não comem o suficiente. Para tal, têm de alternar entre desonestidade e cooperação de forma precisa, para maximizarem os seus ganhos a longo termo. Além do mais, estes limpadores agem de forma diferente com diferentes tipos de clientes, e clientes com os quais tenham tido experiências passadas. Apesar da ecologia comportamental destes mutualismos de limpeza estar já bem estudada, os mecanismos causais destes estão ainda mal percebidos.

Sabe-se que o neurotransmissor Dopamina (DA) tem efeitos modulatórios sobre o comportamento social e o comportamento em geral em vertebrados. É também amplamente conhecido o seu envolvimento na capacidade de decisão, discernimento, memória,

aprendizagem, plasticidade comportamental e percepção do meio ambiente e das suas mudanças. Todas estas capacidades são necessárias à manutenção dos mutualismos de limpeza e sem elas os limpadores não conseguem ajustar o seu comportamento às diferentes situações com que se deparam. Assim, o objectivo do meu trabalho é perceber qual o papel da DA na modulação do comportamento cooperativo e na aprendizagem desta espécie altamente pro-social.

A fim de testar o papel da DA na modulação do comportamento cooperativo, cinco compostos (agonista do receptor D1 – SKF-38393; antagonista do receptor D1 – SCH-23390; agonista do receptor D2 – Quinpirole; antagonista do receptor D2 – Metoclopramida; controlo – solução salina) foram injectados *in situ* em peixes limpadores capturados na *Lizard Island*, Grande Barreira de Coral, Austrália (14°40'S; 145°28'E), procedendo-se à observação do comportamento dos peixes injectados. O bloqueio da transmissão de DA por via do antagonista do receptor D1 aumentou os níveis de investimento na cooperação; o antagonista do receptor D2 teve efeitos semelhantes, mas de forma menos acentuada. Estes efeitos são contrários ao esperado, mas consistentes com a bibliografia existente: a diminuição de DA leva a alterações na capacidade de decisão e adaptação do comportamento; o intenso aumento do investimento na interacção (por via da estimulação táctil) conduz a uma alteração de percepção (défice na capacidade de decisão) por parte limpadores já que previne que estes se continuem a alimentar, procurando exclusivamente o contacto físico (estratégia que prejudica mais o limpador do que propriamente o cliente). Este aumento de investimento pode dever-se a uma excessiva insegurança durante a interacção.

Uma segunda experiência, realizada em cativeiro no Oceanário de Lisboa, foi destinada a investigar o papel da DA na aprendizagem dos limpadores. Em condições controladas, o processo de aprendizagem dos limpadores pode ser explorado usando placas *Plexiglas* de diferentes padrões de cor com comida, substituindo os clientes. Foi simulada uma situação

onde dois clientes se apresentam em simultâneo ao limpador. Nestas situações, o limpador tem de decidir qual dos clientes inspeciona primeiro, correndo o risco do cliente ignorado se ir embora. Como as espécies de clientes variam no seu valor nutricional, este aspecto foi introduzido através da apresentação de comida em apenas uma das placas (placa correcta), forçando os limpadores a aprenderem a identificar a fonte de comida. Os limpadores foram injectados diariamente com os compostos mencionados anteriormente e testados a aprender duas tarefas que diferem em termos de relevância ecológica. A primeira foi uma tarefa visual (ecologicamente relevante), onde os limpadores tinham de aprender a identificar a placa com o padrão correcto. A segunda foi uma tarefa espacial (ecologicamente não relevante), onde os limpadores tinham de aprender a identificar um lado correcto, independentemente do padrão da placa. A manipulação com o agonista do receptor D1 resultou num aumento da velocidade de aprendizagem nas duas tarefas experimentais independentemente da relevância e, ao contrário do previsto, os antagonistas não provocaram uma aprendizagem mais lenta em nenhuma das tarefas. Isto indica que a DA é essencial na aquisição de associações entre estímulos e recompensas, mas que pode estar a actuar em conjunto com outros sistemas neuronais.

Os dados obtidos mostram então que a DA é essencial à aquisição, regulação e manutenção de comportamentos mutualísticos, e contribui para a compreensão das bases fisiológicas e dos mecanismos causais subjacentes aos processos cooperativos entre o peixe limpador *L. dimidiatus* e a sua clientela.

Palavras-chave: Dopamina, neuromodulação, *Labroides dimidiatus*, mutualismo, cooperação, aprendizagem, D1, D2, SKF-38393, SCH-23390, Quinpirole, Metoclopramida

Abstract

In coral reefs, cleaner species such as the cleaner wrasse *Labroides dimidiatus* have the on-going duty of cleaning other reef fish, called clients. Cleaning interactions are a classical example of mutualistic interactions where both parties benefit from it: cleaners remove (eat) ectoparasites and harmful debris off the clients' body surface, consequently contributing for the improved of reef fish health condition and overall ecosystem welfare. Although cleaning mutualisms are already widely studied from a functional point of view, the physiological mechanisms underlying these cooperative interactions are still not well understood. Dopamine (DA) is a neurotransmitter involved in the regulation of social behaviour and heavily influences decision-making. As such, DA is potentially a good candidate modulator of cleaner wrasses' behaviour (to some degree). The present study is one step forward into understanding the exact role of DA in cooperative behaviour modulation.

To do so, the cleaner wrasses' dopaminergic system was manipulated by exogenously administering two agonists and two antagonists and recorded their behaviour *in situ* in Lizard Island, Australia. Cleaner wrasses' cooperative investment increased without increasing cheating events when D1 activity was blocked, probably due to impairment of their judgment and decision-making competence. D2 blockade also increased cooperative investment but not to the same magnitude, and overall DA stimulation had no effects. Additionally, a second experiment (*ex situ*) was performed to test the influence of DA on cleaner wrasses' learning competence. DA stimulation via D1 receptors increased cleaner wrasses' visual and spatial learning, regardless of ecological relevance. The present study provides evidence that DA is involved in neuromodulation of cooperative behaviour as well as learning competence of a coral reef fish.

Keywords: Dopamine, neuromodulation, *Labroides dimidiatus*, mutualism, cooperation, learning, D1, D2, SKF-38393, SCH-23390, Quinpirole, Metoclopramide

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INTRODUCTION

Ecological and ethological components of cleaning mutualisms

Cooperation is defined as any interaction between individuals in which the investment costs are outweighed by the benefits (Dugatkin, 1997). When these interactions are held between different species, specially marine species, they are referred to as mutualisms (which include the cleaning mutualisms; Côté, 2000). The cleaning mutualisms involve two parts: a small and conspicuous *cleaner* (Cheney *et al.*, 2009; Lettieri *et al.*, 2009) and an often bigger sized *client* (Côté, 2000; Grutter, 1995). Species involved in these interactions end up developing behavioural, morphological and/or physiological adaptations to facilitate these interactions (Côté, 2000; Grutter, 2001). The bluestreak cleaner wrasse *Labroides dimidiatus* (Valenciennes, 1839) along with a strict number of species of families *Labridae* and *Gobiidae* present the highest level of specialization in cleaning behaviour, being classified as obligate cleaners (Côté, 2000; Stummer *et al.*, 2004), and establish in small areas or territories known as cleaning stations. A cleaning interaction comprises on the removal of ectoparasites and infected and dead tissue off the clients body, with whom they can interact more than 2000 times in a single day (Grutter, 1995), and can be initiated by clients posing, advertising that they want to be cleaned (Côté *et al.*, 1998). Cleaners can also advertise their services by performing a dance ritual in order to capture their clients attention (Becker et al., 2005; Côté, 2000). At a first glance, this looks like a fair system, where one side benefits from getting rid of harmful parasites, and the other gets access to an easy meal (Bshary and Côté, 2008; Côté, 2000).

However, cleaner wrasses have preference for clients' mucus and scales (Grutter and Bshary, 2003) and often bite those instead of ectoparasites (cheating), which is harmful for their clients, as it requires high energy allocation to replace those lost structures (Grutter and

Bshary, 2004; Grutter, 1997). Because cheating yields positive outcomes to the cheater and negative outcomes to the other party, clients need to resort to control mechanisms (punishment) to ensure cleaner wrasses cooperation/honesty (Bshary and Grutter, 2005; Mills and Côté, 2010). These control mechanisms usually include jolting (clients' rapid body movement), switching between cleaning stations, chasing of a cheating cleaner after it had been dishonest or even eating the cleaner, in case the client is piscivorous (Bshary and Grutter, 2005, 2002; Bshary and Würth, 2001; Mills and Côté, 2010). Cleaner wrasses in turn have strategies of their own and take advantage of their clients' physiological responses. During cleaning interactions, cleaner wrasses roam their clients' body surface searching for ectoparasites, and while doing so, they provide tactile stimulation by massaging their clients with their pelvic fins (Grutter, 2004; Soares *et al.*, 2011). Tactile stimulation has a calming effect on clients because it reduces their stress levels, increases their fitness (Ros et al., 2011; Soares et al., 2011, 2007; Waldie et al., 2011) and manipulates clients into staying longer in the cleaning station (Bshary and Würth, 2001). Furthermore, as this behaviour is considered as cooperative investment, since its time and energy consuming and while performing it there is no food intake (Bshary and Würth, 2001), cleaner wrasses also use it to deceive their clientele (Bshary and Grutter, 2006; Bshary, 2002). Moreover, when in presence of an audience, cleaners provide good service to less valuable clients, which deceives nearby higher value clients into approaching the seemingly honest cleaner to thus be cheated on (Bshary and Grutter, 2006; Bshary, 2002). Finally, tactile stimulation is also used as a conflict management strategy: since predators pose an immediate and more profound threat (death), cleaner wrasses are specially cautious, they usually start the interaction by providing tactile stimulation regardless of the predatory client species and avoid going near their mouth (Grutter, 2004). With non-predatory clients however, tactile stimulation is usually used before or after a cheating event, so they can make the client stay for longer even after being cheated (Bshary and Würth, 2001).

Furthermore, cleaner wrasses need to maximize their gains with a long-term perspective: they would rather cheat than cooperate as it yields higher payoffs; however if they want their clients to return again they must cooperate and provide good service quality. This is highly related to the type of client (Bshary and Grutter, 2006; Grutter, 2004) and on past experience (Salwiczek and Bshary, 2011), which requires acute social recognition, behavioural plasticity, and precise decision-making.

Finally, cleaning mutualisms are of the utmost importance to coral reef ecosystems, being able to drive their entire biodiversity and species richness (Clague et al., 2011; Grutter et al., 2003; Waldie et al., 2011).

Physiological component of cleaning mutualisms

Cleaning behaviour is probably the most studied example of mutualism (Bshary and Côté, 2008) and still, its physiological (causal) components are not yet well understood. Stress is known to be a main variable affecting cleaning interactions: clients with access to cleaning seem to have lower cortisol levels (respond less to stressful conditions as capture and transport) and when their glucocorticoid receptors are blocked (thus maintaining lower cortisol levels) they visit cleaning stations with less frequency (Bshary et al., 2007; Ros et al., 2012, 2011). As for cleaners, cortisol is also able to modulate behavioural changes (Soares *et al.*, *in press*) and increases when cleaners are in contact with predatory clients (Soares et al., 2012a). Other systems also seem to be directly involved in the modulation of cleaning behaviour, such as the neuropeptides arginine vasotocin (influencing the switch that makes the cleaner interact with other species; (Soares et al., 2012b) and the neurotransmitter serotonin which is predictably involved in the regulation of motivation to clean (Paula *et al.*, *in review*).

Dopamine involvement in the modulation of cooperative (*e.g.* cleaning) behaviour

The Social Behaviour Network (SBN) has been described by Newman (1999) as the neural basis of social behaviour in mammals, and since then has been expanded to teleosts and other vertebrate classes (Crews, 2003; Goodson, 2005). This network consists on a group of reciprocally connected brain regions that, in conjunction with steroid hormones (Newman, 1999), are known to regulate multiple forms of social behaviour such as aggression, sexual behaviour and parental care, all seen both in mammals and teleosts (O'Connell and Hofmann, 2012). O'Connel and colleagues (O'Connell *et al.*, 2011) found that each teleost brain region described as a putative homolog for its mammal counterpart (Forlano et al., 2001; Kittelberger et al., 2006; Moore and Lowry, 1998; Wullimann and Mueller, 2004) expresses two dopamine (DA) receptors (D1 and D2), suggesting an important role for DA in social behaviour modulation in teleosts. However, to be adaptive, social behaviour must be rewarding to some extent in order to be reinforced and preserved. Indeed, the mesolimbic reward system and the SBN are two circuits anatomically linked by bidirectional connections between several brain regions, and complement each other by regulating both the evaluation of stimulus salience and the behavioural output. Together, they are better comprehended as a larger, integrated and evolutionary ancient Social Decision-Making (SDM) Network, that modulates and implements responses to incentive stimuli (both social and non-social) and provides animals the ability to make choices (For a full review on the evolution of social decision-making network through vertebrate lineages, see O'Connell and Hofmann 2012). Dopaminergic system is then a highly conserved neurotransmitter system that critically modulates vertebrate behaviour in many ways. For example, DA manipulation is reported to heavily influence decision making (St. Onge and Floresco, 2009; St. Onge et al., 2011, 2010; van Gaalen et al., 2006), conditioned approach to incentive stimuli (Darvas et al., 2014; Flagel et al., 2011a, 2011b, 2007), working memory (Floresco and Magyar, 2006; Takahashi et al., 2012) and

context-appropriate responses (Heimovics and Riters, 2008), all important and crucial features for successful social interactions. As such, DA seems to be the perfect neurotransmitter candidate to test its modulatory effects on this highly social species (the cleaner wrasse *L. dimidiatus*).

Aim of the study

The present work intends to unveil more about the role played by DA in the modulation of the cleaner wrasse *Labroides dimidiatus* cooperative behaviour, as well as in its learning ability, which is especially important in the decision making process (Schultz *et al.*, 1997), and a key for social interactions.

DOPAMINE NEUROMODULATION OF CLEANER WRASSE COOPERATIVE BEHAVIOUR

Introduction

For a long time scientists have considered cooperation a puzzle difficult to explain from an evolutionary point of view, as it goes against Darwin's theory of evolution through natural selection (Brosnan and Bshary, 2010; Bshary and Bergmüller, 2008). A cooperator is an individual who pays a cost for another individual to benefit from its actions (Bshary and Bergmüller, 2008; Nowak, 2006), and expects to be reciprocated, which may not occur. So, why cooperate? Cooperation allows animals to specialize on different functions and provides biological diversity, leading to new levels of social organization (Nowak, 2006) and ideally, the benefits attained from a successful cooperation will outweigh the costs of investment (Bshary and Bergmüller, 2008; Dugatkin, 1997). Although the knowledge on the behavioural mechanisms that lead to the evolution of interspecific cooperation has increased significantly over the years, the knowledge on the proximate mechanisms is still to be acquired (Soares *et al.*, 2010).

Animals have evolved flexible strategies that allow them to respond to the environment by integrating external stimuli with internal physiological cues to produce adaptive behavioural responses (O'Connell and Hofmann, 2012). Since individual fitness depends on displaying adaptive behaviour patterns in a context-appropriate manner, stimulus salience evaluation and animal judgment are the keys to a successful (or otherwise unsuccessful) interaction (O'Connell and Hofmann, 2012; St. Onge and Floresco, 2009). Additionally, physiological and neuronal processes play a vital role on behaviour modulation, as emotional states affect behaviour in several ways and therefore, decision-making. For example, animals are more

prone to cooperate with others when they are in a “positive” mood (Bartlett and DeSteno, 2006).

Dopamine (DA) is a neurotransmitter involved in a variety of neurochemical and neurohormonal modulating actions, such as social behaviour modulation (Baskerville and Douglas, 2010; Heimovics et al., 2009). DA acts by means of synaptic receptors belonging to two receptor families: D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors which differ from each other by their effect over DA-sensitive enzyme adenylate cyclase (AC). When stimulated, D1-like receptors activate this enzyme, whereas D2-like receptors either attenuate or have no effect over it (Callier et al., 2003; Hu and Wang, 1988; Missale et al., 1998). DA is thought to potentiate learning through a reward-related system. The reward system is the neural circuit responsible for external stimulus evaluation and reward-motivated behaviour (Deco and Rolls, 2005; Wickens et al., 2007), by associating actions with their outcomes (Young and Wang, 2004). This system consists on dopaminergic projections from the ventral tegmental area (VTA, midbrain) to several forebrain regions, which is known as the mesolimbic dopaminergic pathway, well described in mammals (Deco and Rolls, 2005; Wickens et al., 2007). Given the importance of this system for the regulation of mammalian behaviour, finding homologous brain regions in teleosts is key to understand how the reward system can modulate fish behaviour. Although debatable, most mammal forebrain regions involved in this pathway have putative homologies in teleosts (Northcutt, 2006, 1995; Portavella et al., 2004; Rink and Wullimann, 2002, 2001). Furthermore, O’Connell and colleagues (2011) found that all these regions express D1 and D2 receptors, which provided neurochemical evidence to support the homologies suggested by previous research, and that the dopamine reward system might function in a similar way in teleost fish and in mammals. Amongst known receptors, D1 and D2/D3 have been shown to be the ones responsible for social behaviour, acting for instance on social status (Martinez et al., 2010; Morgan et al., 2002; Nader et al., 2012), social dominance (Cervenka *et al.*, 2010), sexual-related aggressiveness (Rodríguez-

Arias *et al.*, 1998), partner preference and pair-bonding (Aragona et al., 2006; Gingrich et al., 2000; Smeltzer et al., 2006; Wang et al., 1999).

Additionally, by changing DA activity through D1 and D2 receptor manipulation, several studies have found DA to be involved in reward and risk assessment (St. Onge and Floresco, 2009; St. Onge et al., 2011, 2010), behaviour reinforcement (Berridge and Robinson, 1998; Heimovics et al., 2009), and anticipatory responses to reward-associated stimuli (Heimovics *et al.*, 2009). Furthermore, DA has been shown to bias animal choice via the selective activation of D1 or D2 receptors, promoting risky choices, whereas by blocking these same receptors (henceforth decreasing DA activity) biased to a risk-aversive behaviour (St. Onge and Floresco, 2009). Adding to this, D1 and D2 seem to provide complementary contributions to decision making (St. Onge *et al.*, 2011): D1 receptor activity influences cost/benefit evaluation, helping animals to overcome certain costs associated with larger rewards, in an effort to maximize their gains in a long-term basis; D2 activity however helps to mitigate the immediate impact some rewards or reward-predicting stimulus/events might have over choice bias, ultimately facilitating behavioural adjustment to changing conditions.

DA, amongst several modulating functions, can also work as a teaching signal that helps to predict future events and/or outcomes in various situations (Salamone and Correa, 2012; Schultz, 1998; St. Onge and Floresco, 2009) and facilitates behavioural adjustment, leading to proper decision making. Animals assign different values to stimuli they receive from the environment and events, and the DA system signals (responds) the outcomes as appetitive or aversive (Salamone and Correa, 2012; Schultz, 1998). Through repeated encounters, they learn to associate these stimuli with the usual outcome, while the dopaminergic response progressively transfers from the outcome itself to earlier event-predicting stimuli (Schultz *et al.*, 1995), gradually enabling animals to predict outcomes (Fig. 1).

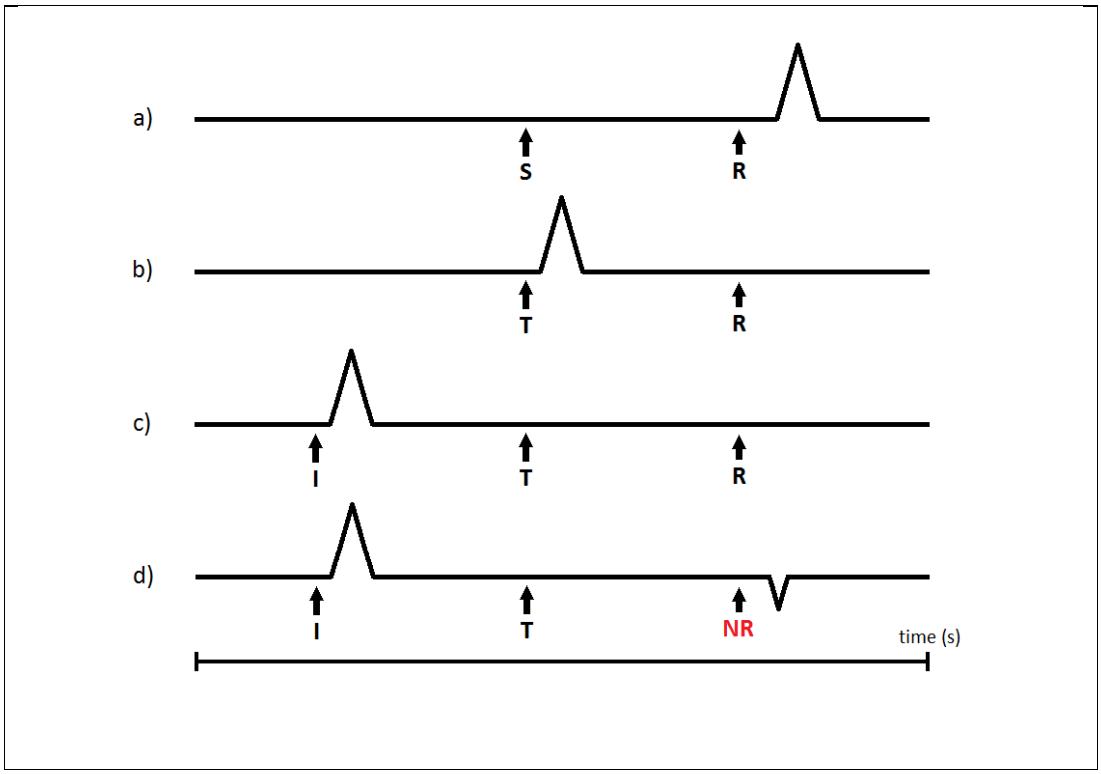

Figure 1 - Evolution of dopamine response (DR) throughout the learning process: a) a stimulus (S) occurs, that later on leads to a reward (R). After the reward is achieved, dopamine neurons (DAn) fire and signal it as something appetitive (spike); b) with repeated encounters/events, this DR progressively transfers from the reward event itself to the earlier stimulus, now a trigger (T) or reward-predicting stimulus; c) the DR can be further associated with an instruction (I), an event or cue signalling the approach of a reward-predicting stimulus that will lead to a reward; d) however when a reward is predicted but fails to occur (no reward – NR), a reward-prediction error is signalled (adapted from Schultz et al., 1995)

However when a certain expected outcome fails to occur, DA signals a prediction error to alert that something changed. In his work, Schultz (1998) explained the DA response (DR) to rewards (appetitive events) with a rather simple equation:

Dopamine Response = Reward Occurred – Reward Predicted

When a new, unpredicted reward occurs following a certain stimulus (Reward Occurred (RO) = 1, Reward Predicted (RP) = 0), DR is positive, signalling an appetitive event. When a reward occurs and it was predicted (RO = 1, RP = 1), DR is neutral. When a reward fails to occur but it was predicted to happen (RO = 0, RP = 1), DR is negative (prediction error). This prediction

error signal enables animals to adjust their predictions and behaviour to new situations, hence working as a teaching signal (for more specific information on how dopamine signals work, see Schultz et al., 1995, 1997; Schultz, 1998, 2002). This equation can be further extended to understand whole Appetitive Events rather than just rewards (Schultz, 1998).

An appropriate model to test the influence of DA is the Indo-Pacific bluestreak cleaner wrasse *Labroides dimidiatus*, which is a cleaner species that enrols in complex cooperative and social interactions (Bshary, 2002; Côté, 2000; Grutter, 2004). This coral reef fish species occupies small territories (known as cleaning stations) and exclusively feed on what it cleans off of other reef fish (called clients), such as dead or infected tissue and ectoparasites (Grutter and Bshary, 2004; Stummer et al., 2004). In turn, clients gain health and fitness benefits from ectoparasite removal (Bshary and Bergmüller, 2008; Côté, 2000), making these cleaner wrasses a key component for coral reef species well-fare (Clague *et al.*, 2011; Waldie *et al.*, 2011). At first glance, one could expect this to be a reciprocally honest system, in which both sides have benefits for cooperating with one another. However, conflict arises as cleaners prefer clients' mucus and scales, which are more nutritious than ectoparasites or dead tissue, but more costly for clients, as energy allocation is necessary to replace them (Grutter and Bshary, 2004; Grutter, 1997). To deal with these conflicts, clients rely on control mechanisms like punishment, either by jolting (rapid body movement), chasing the cheating cleaner or switching cleaning stations (Bshary and Grutter, 2005, 2002; Bshary and Noë, 2003; Mills and Côté, 2010). In rare occasions, predatory clients may eat cleaners in response to their cheating activities (Bshary and Grutter, 2005, 2002; Bshary and Noë, 2003; Mills and Côté, 2010). Cleaners, on the other hand, resort to deceiving and conflict-managing tactics: using their pelvic fins, they stimulate their clients' body by giving massages (called tactile stimulation). Not only they succeed to manipulate clients on staying longer in the cleaning station, but also deceive clients to perceive those cleaners as trustworthy, even after a cheating event (Bshary and Würth, 2001; Grutter, 2004).

This study is then aimed at finding to what extent DA affects cleaner wrasse cooperative behaviour and decision-making strategies in a natural setting.

Material and Methods

Field methods

Field experiments took place on 10 different reefs around Lizard Island (Lizard Island Research Station, Australia, 14˚40'S, 145˚28'E) between September and October 2012, where 50 female cleaner wrasse were tested. All manipulations and observations were performed between 9:00 and 15:00 hours. Cleaner wrasses were selected haphazardly across reefs, while cleaning stations varied in depth (between 1 m and 15 m). All individuals were captured using a barrier and hand net combination, and measured to the nearest mm (TL – Total Length: ranged from 6.0 cm to 8.1 cm), and their body weight was then estimated using a length-weight regression graph (unpublished data). Each focal cleaner was administered, via intramuscular injection, with one of five compounds: saline solution for control (0.9% NaCl); a selective D1 agonist SKF-38393 (D047 – Sigma); D1 antagonist SCH-23390 (D054 - Sigma); selective D2 and D3 agonist Quinpirole (Q102 - Sigma); selective D2 antagonist Metoclopramide (M0763 – Sigma). Injection volumes were always 15 µl per gram of estimated body weight (gbw). This process never exceeded 3 min. Dosages applied were based on previous studies: 5.0 µg/gbw of SKF-38393 (Cooper and Al-Naser, 2006; Dong and McReynolds, 1991; Mattingly et al., 1993), 0.5 µg/gbw of SCH-23390 (Rodríguez-Arias *et al.*, 1999; St. Onge *et al.*, 2011), 2.0 µg/gbw of Quinpirole (Popesku *et al.*, 2010), and 5.0 µg/gbw of Metoclopramide (Brzuska et al., 2004; Mok and Munro, 1998).

SKF-38393 is a selective D1 and partial D5 agonist, that can simulate dopamine activity (Dong and McReynolds, 1991) and can disrupt collective behaviour, such as shoaling (Echevarria *et al.*, 2009). SCH-23390 is a high-affinity selective D1 antagonist with negligible effects on D2 receptors, and slight effects on 5-HT_{2A} receptors (Hyttel *et al.*, 1989). Quinpirole is a selective D2 and D3 agonist (Levant *et al.*, 1993) widely used in a variety of scientific researches related

to D2 receptor manipulation. Metoclopramide, commonly known for its anti-emetic effect via the chemoreceptor trigger zone, is a selective D2 antagonist, acting as a dopamine inhibitor (Albibi and McCallum, 1983; Mok and Munro, 1998). It is expected that D1 antagonist (SCH-23390) and D2 antagonist (Metoclopramide) may contribute to impair cleaner wrasses' ability to make proper decisions by potentially disrupting individual's competence to predict partner signals prior to a reward or appetitive event (refer to Fig. 1) while the D1 agonist (SKF-38393) should boost their activity and induce more bites (cheating)(St. Onge and Floresco, 2009; St. Onge et al., 2011). D2 agonist (Quinpirole) is expected to either reduce cleaner wrasses' overall activity (Wang *et al.*, 1999) or produce a real impairment in choice making (refer to St. Onge et al., 2011).

The order of the treatments was randomized for each dive and all treatments used independent cleaner fish. Once an individual was released it was then observed and videotaped for the next 45 min using a Sony Cyber-Shot DSC-W570 camera in a waterproof housing, always from a distance of 2-3 m.

Behavioural analysis

The videos mentioned before were analysed and the following measurements were noted for each video observation: a) species and TL of each client (visual estimative to the nearest cm) visiting the cleaning station; b) duration (in seconds) of inspection towards each client; c) the number and duration of tactile stimulation (TS) events provided to each client; and d) number of jolts performed by clients, and their behaviour following the jolt.

Statistical Analysis

All selected cleaner wrasses were independent (i.e. were used only once). Measures of cleaner wrasse behaviour towards clients were split into three categories: a) measures of likelihood to engage in cleaning behaviour (motivation to interact); b) measures of interactive investment

(providing of tactile stimulation); and c) measures of cleaner wrasse dishonesty levels. Each of these categories includes several behavioural correlates. Therefore, the likelihood to engage with clientele was measured by: 1) the proportion of clients inspected (calculated as the total number of clients inspected/total number of visits), and 2) the mean duration of inspection (total time of interaction/total number of interactions). Measures of interactive investment were calculated as: 1) the proportion of interactions in which tactile stimulation was used by cleaners (frequency of clients inspected where tactile stimulation occurred/total number of interactions), and 2) the proportion of time cleaners spent providing tactile stimulation to clients (total tactile stimulation duration/total interaction duration). Finally, the measure of cleaners' dishonesty levels was calculated through the frequency of jolts per 100 seconds of inspection. Data were analysed using non-parametric tests because the assumptions for parametric testing were not met. Therefore, each dopaminergic treatment was compared with the control (saline) group using Mann-Whitney U tests, as it is recommended for a small set of planned comparisons (Ruxton and Beauchamp, 2008), followed by Bonferroni corrections, that were applied to account for multiple testing, thus reducing the significance level to $\alpha'=0.0125$.

Ethical commitment

Ethical clearance to work at Lizard Island Research Station (Australian Museum), which involved animal manipulation, was obtained from the University of Queensland Animal Ethics Committee (Native and exotic wildlife and marine animals) – permit nr. SBS/104/10 (project name: "Linking behaviour and physiology in marine cleaning mutualisms"). The use of animals and data collection complied with the laws of Australia, Portugal and Switzerland.

Results

a) Dopamine effects on the likelihood to engage in cleaning behaviour

Cleaner wrasses treated with the D1 antagonist SCH-23390 inspected a significantly higher proportion of clients (calculated as the total number of clients inspected/total number of visits) when compared with the control group (Mann-Whitney U test, SCH-23390 *vs*. Saline: U = 1.0; n1 = 10, n2 = 10, p = 0.0001, Fig. 2), whereas other treatments did not produce any significant effects (SKF-38393 *vs*. Saline: U = 23.0; n1 = 10, n2 = 9, p = 0.08; Quinpirole *vs*. Saline: U = 24.0; n1 = 10, n2 = 10, p = 0.03; Metoclopramide *vs*. Saline: U = 17.0; n1 = 10, n2 = 10, p = 0.02, Fig. 2).

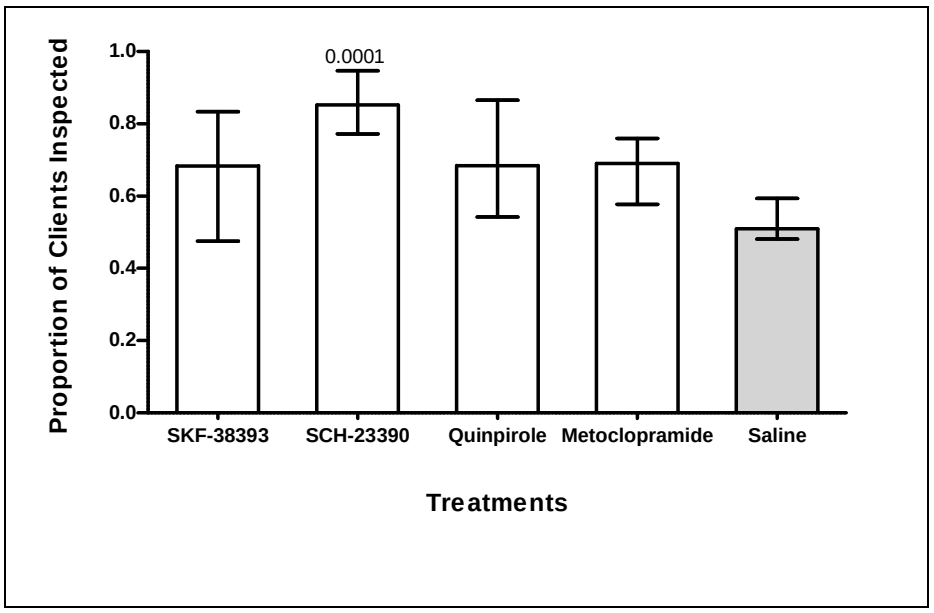

Figure 2 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on cleaner fish *Labroides dimidatus* likelihood to engage in cleaning behaviour: Proportion of Clients Inspected (calculated as the total number of clients inspected/total number of visits). Medians and interquartile ranges are shown. Probability value (shown above bar) refers to Mann-Whitney U tests affecting each dopaminergic treatment against the reference (saline) group, for a total sample size of 10 individuals.

Regarding the duration of inspection, cleaner wrasses treated with D1 antagonist SCH-23390 spent more time inspecting their clientele when compared with control (SCH-23390 *vs*. Saline:

U = 6.0; n1 = 10, n2 = 10, *p* = 0.001, Fig. 3), while none of the remaining treatments were found to have a substantial effect on client inspection duration (SKF-38393 vs. Saline: U= 35.0; n1= 10, n2= 9, *p*= 0.45; Quinpirole vs. Saline: U= 44.0; n1= 10, n2= 10, *p*= 0.47; Metoclopramide vs. Saline: U= 32.0; n1= 10, n2= 10, *p*= 0.32, Fig. 3).

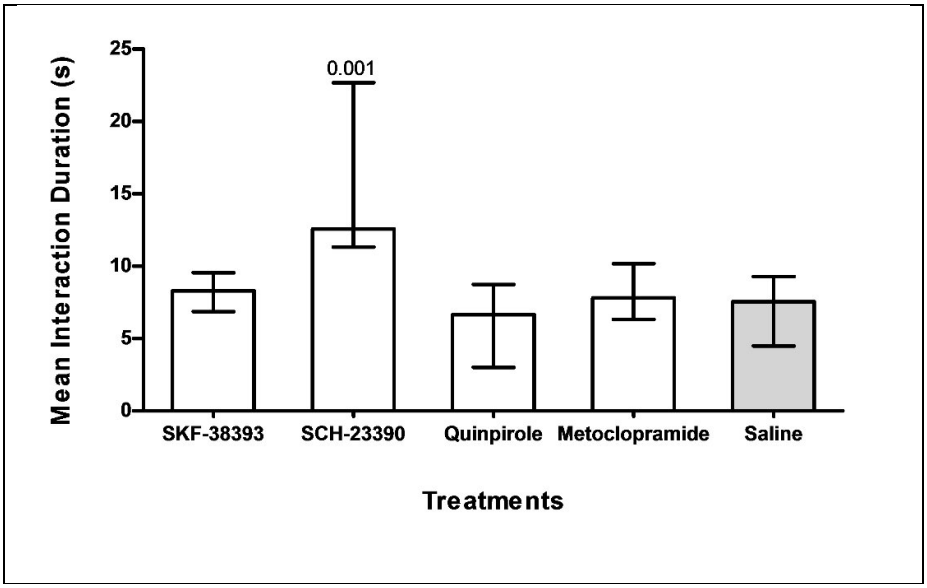

Figure 3 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on cleaner fish Labroides dimidatus likelihood to engage in cleaning behaviour: Mean Interaction Duration (total time of interaction/total number of interactions). Medians and interquartile ranges are shown. Probability value (shown above bar) refers to Mann-Whitney U tests affecting each dopaminergic treatment against the reference (saline) group, for a total sample size of 10 individuals.

b) Dopamine effects on tactile stimulation of clients

Both treatments with D1 antagonist SCH-23390 and D2 antagonist Metoclopramide were found to increase the proportion of events in which cleaners provided tactile stimulation to their clients (SCH-23390 *vs*. saline: U = 8.0; n1 = 10, n2 = 10, *p* = 0.001; Metoclopramide *vs.* saline: U = 8.0; n1 = 10, n2 = 10, *p* < 0.002, Fig. 4) but the same was not observed with the remaining compounds (SKF-38393 *vs.* saline: U = 18.0; n1 = 10, n2 = 9, *p* = 0.03; Quinpirole *vs.* saline: U = 42.5; n1 = 10, n2 = 10, *p* = 0.39, Fig. 4).

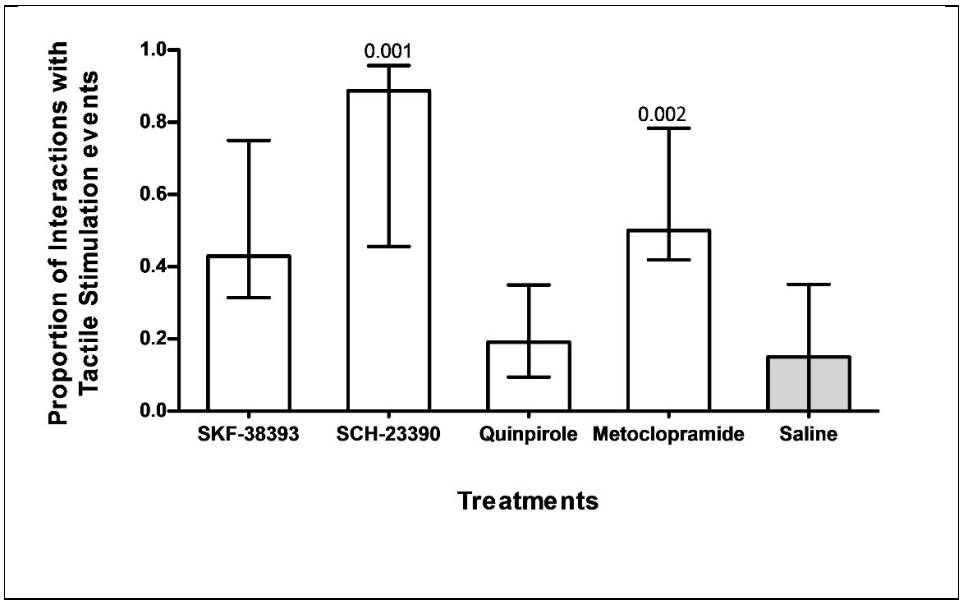

Figure 4 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on the proportion of interactions where tactile stimulation occurred: Proportion of Interactions with Tactile Stimulation events (Frequency of clients inspected where Tactile Stimulation occurred/Total number of interactions). Medians and interquartile ranges are shown. Probability values (shown above bars) refer to Mann-Whitney U tests affecting each dopaminergic treatment against the reference (saline) group, for a total sample size of 10 individuals.

Compared to the control, individuals treated with D1 antagonist SCH-23390 spent more time of their inspection providing tactile stimulation to their clients (SCH-23390 *vs.* saline: U = 14.0; n1 = 10, n2 = 10, $p = 0.005$, Fig. 5), while none of the remaining treatments were found to have an effect on this measure (SKF-38393 *vs.* saline: U = 35.0; n1 = 10, n2 = 10, $p = 0.45$; Quinpirole *vs.* Saline: U = 49.0; n1 = 10, n2 = 10, $p = 0.70$; Metoclopramide *vs.* saline: U = 28.0; n1 = 10, n2 = 10, $p = 0.18$, Fig. 5).

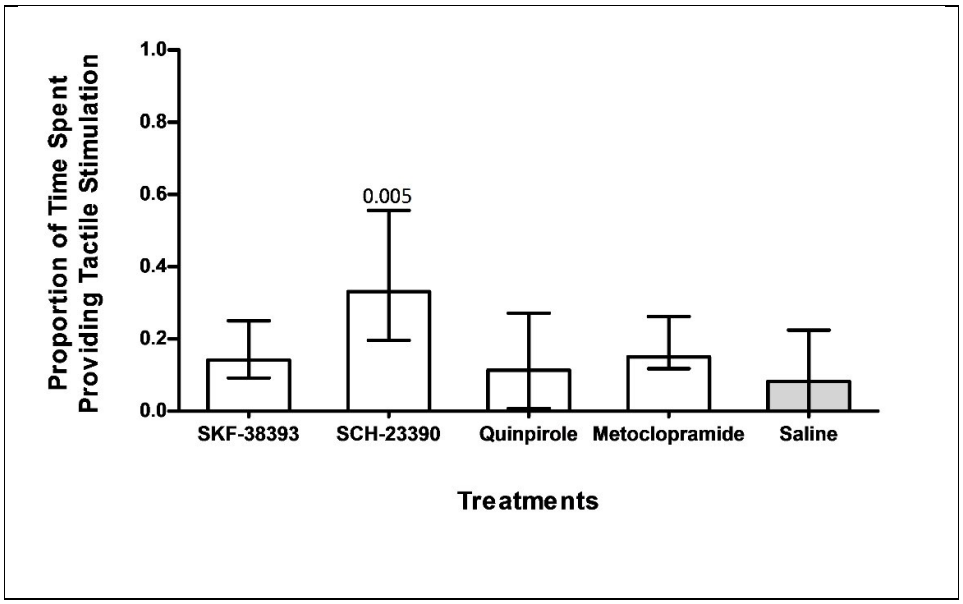

Figure 5 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on the proportion of time cleaners spent providing tactile stimulation (total tactile stimulation duration/total interaction duration). Medians and interquartile ranges are shown. Probability value (shown above bar) refers to Mann-Whitney U tests affecting each dopaminergic treatment against the reference (saline) group, for a total sample size of 10 individuals.

c) Dopamine effects on cleaner wrasses dishonesty levels

Considering jolt frequency of clients, no effects of the given treatments were found on the cleaners' behavioural response (SKF-38393 *vs.* Saline: U = 23.0; n1 = 10, n2 = 9, $p = 0.08$.; SCH-23390 *vs.* Saline: U = 33.0; n1 = 10, n2 = 10, $p = 0.22$; Quinpirole *vs*. Saline: U = 52.5; n1 = 10, n2 = 10, $p = 0.86$; Metoclopramide *vs*. Saline: U = 23.0; n1 = 10, n2 = 10, $p = 0.08$).

Discussion

The results presented in the previous section show that DA manipulation is able to modulate cleaner wrasses' cooperative behaviour. Below is a summarized representation of the effect each compound treatment had on each behavioural measure, when compared to control (Table 1).

Table 1 - Summarized representation of the effects of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on cleaner wrasse *Labroides dimidiatus* cooperative behaviour, when compared with the control group

Behaviour	Target analysed	SKF-38393	SCH-23390	Quinpirole	Metoclopramide
Likelihood to engage in cleaning behaviour					
a) Proportion of Clients Inspected	Cleaner wrasse	↔	↑	↔	↔
b) Mean Interaction Duration	Cleaner wrasse/Client	↔	↑	↔	↔
Cooperation Investment					
a) Proportion of Interactions with Tactile Stimulation events	Cleaner wrasse	↔	↑	↔	↑
b) Proportion of Time spent Providing Tactile Stimulation	Cleaner wrasse	↔	↑	↔	↔
Cooperative level					
a) Client Jolt Frequency	Client	↔	↔	↔	↔

Note: Arrows indicate the effect, relative to saline, on behaviours of interest: '↑'denotes an increase in display; '↔' indicates no effect detected.

Effects of D1 and D2 receptor blockade

Cleaner wrasses treated with D1 antagonist (SCH-23390) increased behavioural displays on all four behavioural measures, when compared to the control group. Indeed, these individuals were more likely to engage in cleaning interactions, and were also spending more time with each client, as seen by a significantly increase in the proportion of clients inspected and in the

mean interaction duration (Table 1). This was contrary to first predictions as D1 blockade impairs DA transmission (St. Onge and Floresco, 2009), which is essential for signalling events that may prompt seeking behaviour; so motivation was expected to decrease. Moreover, D1 blockade not only resulted in an increase of the amount of tactile stimulation provided, when compared to the control group, but also in spending a significantly higher portion of their interaction time providing tactile stimulation (Table 1). Rises in these two behavioural measures indicate that cleaner wrasses are investing more time and effort in each interaction and each client they encounter but getting little food reward. Moreover, because the values related to the provision of tactile stimulation were prevalent (close to 90%) means that almost every client inspected received physical contact. However, these clients were receiving physical contact without an increase of cheating by the cleaners (no differences in client jolt frequency).

Focal cleaner wrasses then inspected and engaged in physical contact with the majority of clients inspected, which deviates from the more selective and deceptive behaviour this cleaner species usually demonstrates (Bshary and Grutter, 2006; Bshary and Würth, 2001; Bshary, 2002; Grutter, 2004; Mills and Côté, 2010) as well as the behavioural plasticity they show with different types of clientele (Salwiczek and Bshary, 2011). This ability to adjust behaviour should arise from being able to predict based on previous experiences (St. Onge *et al.*, 2010). Indeed, cleaner wrasses rely on their competence to predict to some extent how clients will behave or how an interaction will proceed, as their own survival (food intake) depends on their decisions (Côté, 2000). Cleaner wrasses are known to have more than 2000 interactions a day (Grutter, 1995), so most of their decisions come from predictive associative behaviour. It is also known that cleaner wrasses can remember aspects of past interactions (Salwiczek and Bshary, 2011) and can categorize their clients through their physical characteristics (Bshary et al., 2002; Grutter and Bshary, 2004), therefore being able to identify clientele that are dangerous when cheated on and clients that are safe to cheat on, based on previous encounters. This

identification should in theory also dictate to whom cleaner wrasses give tactile stimulation to (in natural conditions), which differs with the type of client and the type of situation presented (Bshary and Würth, 2001; Grutter, 2004). These results suggest that by manipulating the DA system, cleaner wrasses behavioural adjustments become disrupted as D1 blocked cleaner wrasses were rarely seen removing parasites (cleaning) and were mostly providing tactile stimulation.

Although tactile stimulation is beneficial for clients (Ros *et al.*, 2011; Soares *et al.*, 2011) and is also used as a way to deceive clients (Bshary and Würth, 2001; Bshary, 2002), it is a behaviour that has associate costs , since cleaner wrasses don't get to eat while providing tactile stimulation (Bshary and Noë, 2003; Poulin and Grutter, 1996; Soares et al., 2011). However, in order for cleaner wrasses to maximize their gains in a long-term basis, they have to balance cheating and investment, since cheating yields a higher reward (mucus), almost always followed by tactile stimulation (Brosnan and Bshary, 2010; Bshary and Würth, 2001). The unusual non-selective display of tactile stimulation paired with the absence of client jolts observed in D1 blocked individuals points to a disruption in their ability to adjust behaviour according to the situation and further demonstrates a tendency to lower risky choices in a potential setting of perceived insecurity (Schweimer and Hauber, 2006; St. Onge and Floresco, 2009; St. Onge et al., 2010). Thus, disruption of DA transmission can lead to abnormal reinforcement or event-predicting signals, compromising their judgment and sensitivity to benefits and leading to impaired decision-making.

D2 blockade produced similar results as those of D1 blockade regarding the proportion of interactions where tactile stimulation events occurred. There were no effects over other measures, which is quite interesting, given that Metoclopramide is a powerful D2 antagonist (Albibi and McCallum, 1983). Yet, it is not an unexpected effect given that similar to D1 blockade, D2 blockade is reported to induce risk aversion behaviour in other animal models

(St. Onge and Floresco, 2009; St. Onge et al., 2010), and tactile stimulation, as mentioned above, is used as a conflict management strategy.

The fact that D2 blockade potentiated the increase of tactile stimulation events but not the amount of time spent providing it, and D1 blockade produced a heavier impairment over cleaner wrasses overall judgment, leads to the conclusion that D1 and D2 blockades produced rises regarding cooperative investment, yet with different magnitude. Figure 6 is a hypothesized representation of what might be happening in the cleaner wrasses dopaminergic reward system. The first situation (Fig. 6a) is the final stage of the learning process. Here, the animal already knows that when an Instruction (I) or cue occurs, a certain Trigger action (T) or behaviour will lead to a certain Reward (R) and hence, the DA neurons (DAn) signal the Instruction (reward-predicting signal) that ultimately leads to such reward. If this theory is extrapolated to this cleaner species system, the Instruction (I) would be the client posing, signalling its availability and willingness to interact. The Trigger (T) needed could be tactile stimulation, which gives cleaner wrasses access (or a prolonged access) to their clients' body surface (R). So when D1 receptors are blocked (Fig. 6c), DA transmission is disrupted and DA signal either fails to occur or becomes too weak, ultimately leading the cleaner wrasse to not being able to retrieve the information needed from their actions. This can therefore explain why D1 blockade promotes cooperative investment: cleaner wrasses provide tactile stimulation to their clientele to gain access to their body surface; however, they are unable to determine whether they achieved their goal or not, since their ability to gather, evaluate and integrate information is impaired. Consequently, most of the time is spent investing in their interactions, without being able to receive any output from it and adjust their behaviour to changing situations. When D2 receptors are blocked (Fig. 6b), the initial reward-predicting stimulus fails to occur, however they are able to receive the output of their actions and integrate this information, therefore leading to increases in the proportion of tactile stimulation events, but not in the proportion of time spent providing it.

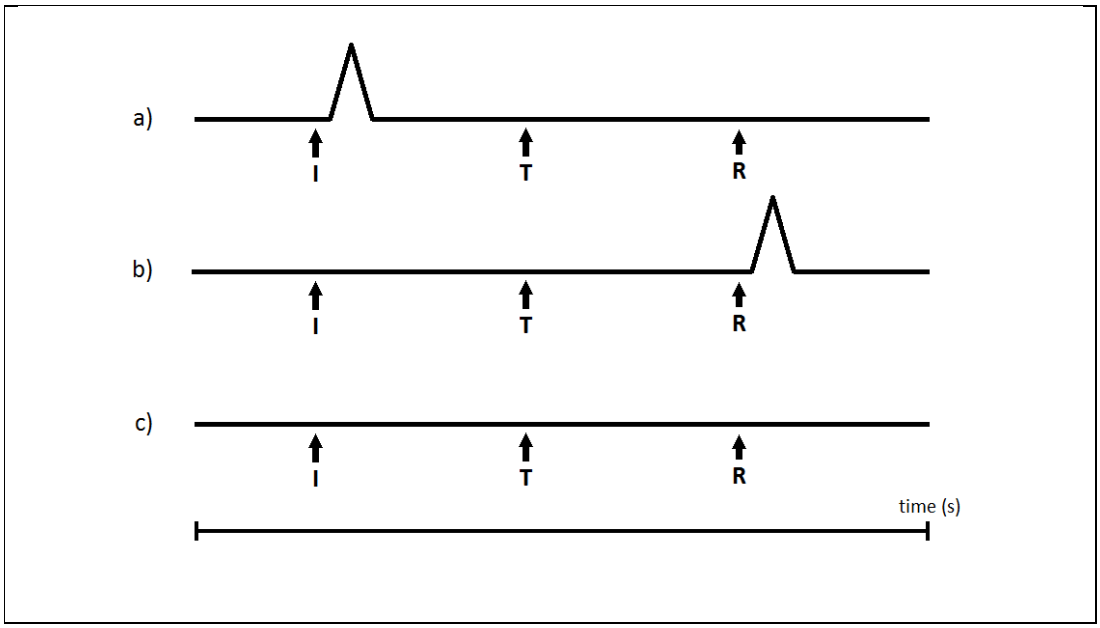

Figure 6 - Hypothesized representations of DA manipulation on reward signaling in a cleaner wrasse – a) regular reward-predicting stimulus (I) that precedes a trigger (T) that leads to a reward (R); b) With DA transmission impaired by D2 antagonist administration, the prediction signal (I) that should fire is disrupted and fails to occur. However, DA still signals the reward achieved and hence cleaner wrasses stop providing tactile stimulation; c) DA transmission is disrupted by D1 antagonist administration, causing the DA signaling to fail, even when the reward is achieved, hence the continuous providing of tactile stimulation, even when cleaner wrasses already have access to their client's body surface (hypothetical reward)

Effects of D1 and D2 receptor stimulation

Neither D1 nor D2 receptor stimulation produced significant effects on cleaner wrasses behaviour (Table 1). DA receptor stimulation has been demonstrated to significantly alter decision making by enhancing DA transmission, where for example D1 stimulation produced increases in risky behaviour (St. Onge and Floresco, 2009; St. Onge et al., 2010) and facilitated effort-based decision making (Schweimer and Hauber, 2006). Thus, D1 agonist treated cleaner wrasses were expected to show an increase in the motivation to interact and also on cleaners' cooperative levels. It was also expected for D2 agonist treated animals to either be less active,

since Quinpirole (D2 agonist) is reported to affect locomotion (Allison et al., 1995; Mattingly et al., 1993); or to produce a perceptive shift in cost/benefit evaluation. For example, D2 agonist treated rats had their choices biased towards small rewards (low risk choice) when the probability of obtaining a large reward (risky choice) was 100%, and towards large rewards when they were less likely to be obtained (St. Onge et al., 2011).

Concluding remarks

Here is shown that DA pathways relevant to cleaner wrasse behaviour modulation mostly act through D1 receptor activity. D1 blockade seems to contribute to disrupt DA transmission and impair the reward signalling, weakening cleaner wrasses' ability to properly evaluate their surroundings, to receive and integrate output from their actions, which ultimately reduces their sensitivity to benefits. The D2 blockade also disrupted DA transmission, but on a different magnitude since cleaner wrasses appear to be able to receive output from their own actions leading to no effects over the other measures, probably due to D1 receptor activity. D1 or D2 stimulation did not produce any significant effects over cleaner wrasses behaviour; increases in DA transmission do not seem to significantly interfere with decision making in these animals (in a natural setting). These results are consistent with the development of a predictive reward signalling, animal perception and subsequent choice. Increases in DA transmission (D1 and D2 stimulation) will not yield any gains or significant effects over the reward signalling or reward predicting signal. Decreases however, will weaken this signal and either cause it to fail completely (D1 blockade) or cause delay (D2 blockade).

DOPAMINE NEUROMODULATION OF CLEANER WRASSE LEARNING COMPETENCE

Introduction

The capacity to anticipate (or predict) is crucial for deciding among different courses of action available (Schultz *et al.*, 1997), as different options entail different final outcomes. Through repeated encounters, animals learn to associate certain stimuli or events to their corresponding final outcomes (O'Doherty, 2004; Schultz, 1998; Young and Wang, 2004) and are thus able to remember previously learned associations in current interactions, potentiating better decision-making (Berridge and Robinson, 1998). The ability to anticipate should become especially important when occurring in social or cooperative contexts, which often comprise complex networks of decisions based on the several intervenient actions (Heimovics and Riters, 2008). Indeed, animals can predict a plenitude of aspects from their surroundings, from spatial discrimination to physical cues (Schultz *et al.*, 1997), and one of the simplest, yet useful predictions animals make is how much rewarding an event will be and when will it occur. Rewards can be perceived as positive values attributed by animals to objects, events, behaviours or internal states (Schultz, 2002; Schultz et al., 1997). The value given to these rewards can for instance elicit approach and consummatory behaviour (James *et al.*, 1989; Phillips *et al.*, 2008) and act as positive reinforcement, prompting certain behaviours to occur (DeWitt, 2014; Phillips et al., 2008; Schultz, 1998). However, attributed reward values depend on the animal's internal state (baseline status) at the moment of stimulus occurrence and their previous experience with that same stimulus (Schultz *et al.*, 1997). Animals' baseline status should also be integrated and reward attribution should also be functionally integrated in their ecological settings.

Studies concerning teleost fish (Zebrafish) have demonstrated that a Dopamine (DA) level increase facilitates cognitive functions and is responsible for an increase in learning speed (Eddins *et al.*, 2009). DA is thought to potentiate learning through the reward system, a neural circuit responsible for external stimulus evaluation, reward-motivated and goal-directed behaviour (Deco and Rolls, 2005; Heimovics and Riters, 2008; Wickens et al., 2007) and consists on dopaminergic projections from the ventral tegmental area (VTA, midbrain) to several forebrain regions, commonly known as the mesolimbic dopaminergic pathway, well described for mammals (Deco and Rolls, 2005; Wickens et al., 2007) and teleosts alike (O'Connell and Hofmann, 2012; O'Connell et al., 2011). Indeed, DA signalling has been widely accepted to contribute to reward-related learning and reward-related processes in general, and DA neurons (DAn) in certain brain regions have long been associated with the processing of reward (O'Connell and Hofmann, 2012; O'Connell et al., 2011; Schultz et al., 1997), yet its exact role is still not yet well understood (Saunders and Robinson, 2012). There are currently two hypotheses regarding the role played by DA in these processes. One is that DA is used to update the predictive value of a Conditioned Stimulus (CS) during associative learning, as to allow behaviour adjustment. The second refers to DA regulation of the attribution of incentive value to CS, which mediates reward salience and henceforth elicits approach (Darvas et al., 2014; Flagel et al., 2011b; Saunders and Robinson, 2012). It is certainly hard to determine whether DA mediates the predictive or motivational properties of the reward-associated stimuli as these two features are regularly acquired simultaneously (Darvas *et al.*, 2014), are strongly associated and usually change together (Saunders and Robinson, 2012). However, individuals vary in the extent to which they attribute motivational values to reward cues. This variation can be assessed by observing the behavioural responses to CS to dissociate which components of reward DA is mediating (Flagel et al., 2011b).

DA signalling enables animals to perceive stimuli as appetitive (rewarding) or aversive, henceforth increasing and maintaining the frequency of behaviours that lead to appetitive

outcomes or decreasing the frequency of behaviours that lead to negative outcomes (Dayan and Balleine, 2002; DeWitt, 2014). Furthermore, DA works as a teaching signal by signalling changes in predicted outcomes: during the process of learning, animals are presented with neutral stimuli before a reward being presented (Unconditioned Stimulus – US) and DAn respond upon reward display, signalling it as appetitive; through repetition, animals learn to expect a reward after the neutral stimulus, now a CS, hence react before the reward is presented (Conditioned Response – CR). Physiologically speaking, DAn progressively switch their response from the moment the reward is presented to the moment where the CS occurs, thus explaining the premature reaction (Heimovics et al., 2009; Schultz, 2006; Schultz et al., 1997). However, when a predicted event fails to occur, or the magnitude and/or probable time of occurrence is altered, DAn signal a prediction error (by decreasing DA levels), alerting the animal that the expected outcome is different from the one predicted, thus leading to behavioural adjustment to match the new conditions (St. Onge et al. 2010; Maximino & Herculano 2010; Schultz 1998; refer to previous section, Fig.1). Thus, shifts in DA levels help animals to learn in a trial-and-error basis, by continuously making predictions and comparing predicted and actual events/outcomes (Fiorillo *et al.*, 2003).

Highly social animals need to be able to predict and adjust behaviour on a regular basis as survival depends on such interactions (Heimovics and Riters, 2008). The Indo-Pacific bluestreak cleaner wrasse *L. dimidiatus* is a cleaner species that enrols in complex cooperative and social interactions (Bshary, 2002; Côté, 2000; Grutter, 2004). These coral reef fish exclusively feed on what they clean off of other reef fish (called clients), such as dead or infected tissue and ectoparasites (Grutter and Bshary, 2004; Stummer et al., 2004). Clients gain health and fitness benefits from these cleaning interactions, and often seek these cleaner wrasses several times a day (Côté, 2000; Ros *et al.*, 2011). This, however, is not a fair system, since both parties can have different goals. Clients want cleaner wrasses to cooperate and remove ectoparasites, yet cleaner wrasses prefer to bite off mucus and scales that are far more nutritious (Grutter and

Bshary, 2003). This non-cooperative behaviour is called “cheating”, and it’s disadvantageous for clients as energy allocation is necessary to replace those important structures that shield the animal of external adversities (Grutter and Bshary, 2004; Grutter, 1997). Cleaner wrasses rely on their ability to discriminate between situations (knowing when to cheat, deceive or cooperate) and to solve problems inherent in high valuing current benefits and discounting both imminent and delayed future benefits (Grutter and Bshary, 2003). Hence, an acute learning ability and behavioural plasticity are key, since switching between cooperation and deception and responding according to context is needed to maximize their long-term gains.

In laboratory conditions, cleaner wrasses’ learning process can be researched by using Plexiglas plates offering food as replacement of clients, where different colour patterns allow visual identification of plates. As such, this study is aimed at finding to what extent DA manipulations interfere with the learning speed of these animals, in accordance to two different ecologically relevant cues.

Materials and methods

Experiments were conducted at the Oceanário de Lisboa fish housing facilities (Lisbon, Portugal), where a total of 10 wild caught cleaner wrasses originated in the Maldives and directly imported to Portugal by a local distributor were used. The fish were kept in individual aquaria (100 x 40 x 40 cm), combined in a flow through system that pumped water from a sump tank (150 x 50 x 40 cm). Nitrite concentration was kept to a minimum (always below 0.3 mg/L), and each tank contained an air supply and a commercial aquarium heater (125 W, Eheim, Jäger). Small PVC pipes (10-15 cm long; 2.5 cm diameter) were used as shelter. Experiments were conducted between March and October 2013. Cleaner fish were previously taught to feed on mashed shrimp from a plexiglas plate (white colour), as a way to simulate the wild feeding habit of picking off their clientele, and took 1-3 days to learn. Different colour patterns were randomly assigned to every plexiglas plate, and a different pair was used for each compound treatment (Fig. 7).

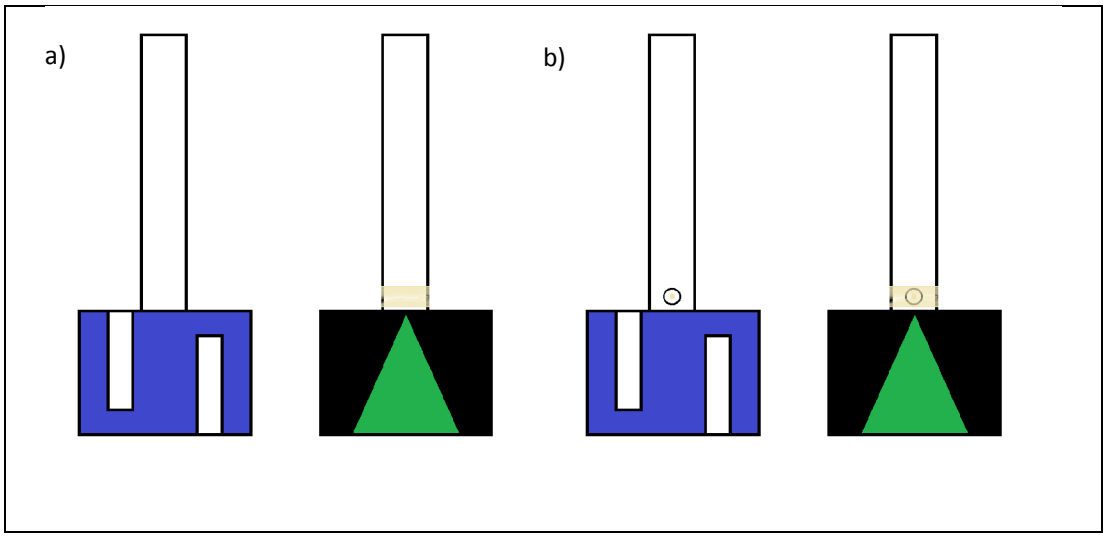

Figure 7 - An example of two pattern colour plates used in the experiment as seen from the front (a) and back (b): left blue plate is the “correct” choice; right black/green plate is the “incorrect” choice. These were assigned to one compound treatment, and presented with the front view to the cleaner fish.

Here, a situation which regularly occurs under natural conditions was simulated where two clients seek service simultaneously (Bshary and Côté, 2008). In such situations, cleaner wrasses have to choose which client to inspect first, with the risk that the ignored client will leave (Bshary and Schäffer, 2002). Client species differ with respect to their nutritional value, *i.e.* parasite load (Bansemer et al., 2002; Grutter, 1994; Soares et al., 2008), and this aspect was introduced by offering food on one plate only. Thus, cleaners had to learn to identify the food source. Two experiments were conducted that differed in the cues presented to identify the correct choice. In the first experiment, Cue discrimination task, cleaner wrasses had to identify the plate that consistently provided food irrespectively of its location, which is a situation ecologically relevant. In the second experiment, Spatial discrimination task, they had to identify which side would be consistently providing food, which is a not-relevant ecological task. Each individual was used in both experimental tasks, and tested with the five compound treatments.

a) Cue discrimination task

This experiment consisted on several sessions (up to eight) in which pairs of plates with different patterns were presented to the cleaner fish (Fig. 8): one was deemed to be the one from which cleaner needed to start eating first (correct pattern) and the second that could wait but had no food/reward available (incorrect pattern). All plates were initially introduced to cleaners the day before the start of the experiments, with a small piece of prawn in the back. Cleaners were submitted to one session per day, in alternate days. Each session was composed of 10 trials, until the individual had learned (three successive sessions in which the individual chose the correct pattern plate in at least 7 out of 10 trials, two successive sessions in which the cleaner chose the correct pattern plate at least 8 out of 10 trials or just one session with at least 9 out of 10 trials). The first trial began 10 minutes after the compound treatment and consisted on successive presentation of the two pattern colour plates every 10

minutes until the completion of 10 trials. The “correct” pattern plate had available prawn whilst the “incorrect” pattern plate had inaccessible prawn (covered with transparent tape) at the back, so out of sight for cleaners during the choice process. Experimental individuals would then have complete access to both plates but while a correct choice would enable both plates to stay inside the aquarium, an incorrect choice would consequently have the correct plate (and the only one with accessible food) be immediately removed, preventing cleaners to eat the food item.

b) Side discrimination task

This task consisted on several sessions, with a similar layout as the previous experimental task, with pairs of plates with colour patterns being displayed to the cleaner fish, each pair associated with a compound treatment (Fig. 8). This task consisted on learning that there was a correct side to start eating from (spatial learning), instead of a correct colour pattern plate (cue learning), which could be inserted in any side of the aquarium (above). Plates were again initially introduced to cleaners with a small piece of prawn in the back, one plate at a time, and one day before the beginning of experiments. Cleaners were (as above) submitted to one session a day, in alternate days, until a maximum of eight sessions. Before each session, individuals were injected with one of a total of five randomly assigned compound treatments. A session would start 10 minutes after the injection, and plates were displayed in 10 minute intervals, for a total of 10 trials per session, until the individual had learned (three successive sessions in which the individual chose the correct pattern plate in at least 7 out of 10 trials, two successive sessions in which the cleaner chose the correct pattern plate at least 8 out of 10 trials or just one session with at least 9 out of 10 trials). The “correct” side was chosen randomly and was maintained between trials and sessions but it changed between compound treatments. The “correct” side plate had available prawn while the “incorrect” side plate had inaccessible prawn (covered with a transparent tape). Experimental individuals would then

have complete access to both plates but while a correct choice would enable both plates to stay inside the aquarium, an incorrect choice would consequently have the correct side plate (and the only one with accessible food) be immediately removed, preventing cleaners to eat the food item.

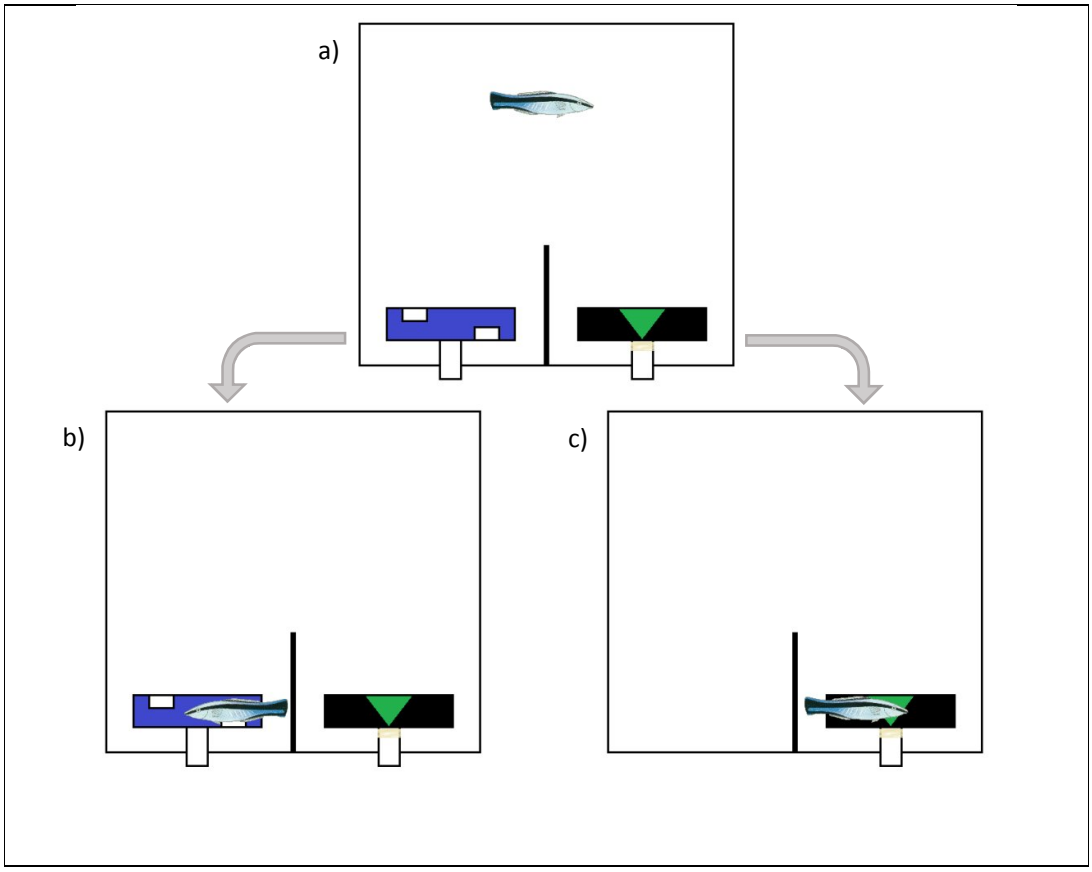

Figure 8 - Upper view of one experimental aquarium. Both experiments used the same layout. Initially (a), both patter colour plates are presented at the same time, for the cleaner fish to choose. When cleaner fish choose the correct plate/side (b), both plates are left inside the aquarium for the cleaner to inspect. When cleaner fish choose the incorrect plate/side (c), the correct plate/correct side plate is removed, leaving the cleaner fish with only the incorrect plate/incorrect side plate to be inspected.

c) Hormonal treatment

Cleaners were weighed before the onset of the experiment so that injection volume could be adjusted to body weight. The following compound treatments were used: saline solution for control (0.9% NaCl); a selective D1 agonist SKF-38393 (D047 – Sigma); D1 antagonist SCH-

23390 (D054 - Sigma); selective D2 and D3 agonist Quinpirole (Q102 - Sigma); selective D2 antagonist Metoclopramide (M0763 – Sigma). Injection volumes were always 15 µl per gram of estimated body weight (gbw). This process never exceeded 3 min. Dosages applied were based on previous studies: 5.0 µg/gbw of SKF-38393 (Cooper and Al-Naser, 2006; Dong and McReynolds, 1991; Mattingly et al., 1993), 0.5 µg/gbw of SCH-23390 (Rodríguez-Arias *et al.*, 1999; St. Onge *et al.*, 2011), 2.0 µg/gbw of Quinpirole (Popesku *et al.*, 2010), and 5.0 µg/gbw of Metoclopramide (Brzuska et al., 2004; Mok and Munro, 1998).

SKF-38393 is a selective D1 and partial D5 agonist, that can simulate dopamine activity (Dong and McReynolds, 1991). SCH-23390 is a high-affinity selective D1 antagonist with negligible effects on D2 receptors, and slight effects on 5-HT_{2A} receptors (Hyttel *et al.*, 1989). Quinpirole is a selective D2 and D3 agonist (Levant *et al.*, 1993) widely used in a variety of scientific researches related to D2 receptor manipulation. Metoclopramide, commonly known for its anti-emetic effect via the chemoreceptor trigger zone, is a selective D2 antagonist, acting as a dopamine inhibitor (Albibi and McCallum, 1983; Mok and Munro, 1998)

D1 antagonist (SCH-23390) and D2 antagonist (Metoclopramide) are expected to impair cleaner wrasses learning speed, since interference with DA transmission is reported to impair the acquisition of conditioned approach responses (Salamone and Correa, 2012). However, D2 antagonists are reported to not having an affect over working memory, which is an important aspect of learning (Takahashi *et al.*, 2012); D1 agonist (SKF-38393) should improve learning speeds, as increasing DA transmission is reported to enhance learning of a conditioned response to visual stimuli (Harley, 2004) and spatial learning (Eddins *et al.*, 2009) and to facilitate working memory (Takahashi *et al.*, 2012)

d) Statistical analysis

The same cleaners were used in all treatment groups in both tasks. Data were analysed using

planned comparisons of least squares means in order to compare each dopaminergic treatment with the control (saline) group. All statistical tests shown in this study were two tailed. Although it would be interesting to compare both tasks, these are not comparable because the second task involves reversal learning.

Results

a) Cue Discrimination Task

Cleaners injected with the D1 agonist SKF-38393 required significantly less sessions to accomplish the learning of a cue discrimination task (Planned comparisons: SKF-38393 *vs.* Saline: $F_{1,9} = 6.69$, $p = 0.03$, Fig. 9), while no significant effects were found to cleaners learning speed by the remaining compounds (SCH-23390 vs Saline: $F_{1,9} = 2.43$, $p = 0.15$; Quinpirole *vs.* Saline: $F_{1,9} = 0.02$, $p = 0.90$; Metoclopramide *vs.* Saline: $F_{1,9} = 0.22$, $p = 0.65$, Fig. 9).

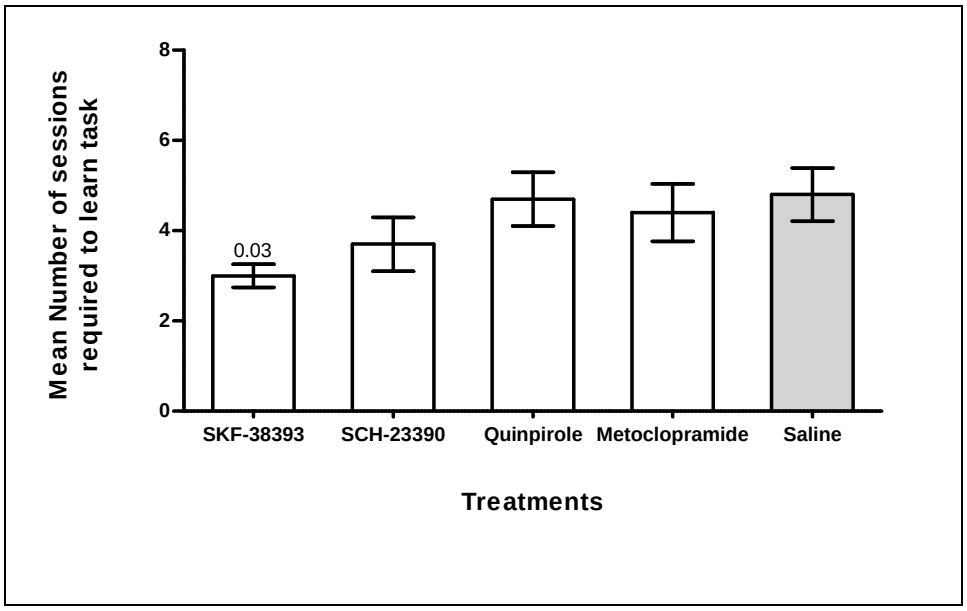

Figure 9 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on cleaner fish *L. dimidiatus* learning speed of a cue discrimination task. Means are shown ± SEM. Probability value (shown above bar) refer to planned comparisons of least squares means effect of each dopaminergic treatment group against the reference (saline) group. Samples sizes were n = 10.

b) Side Discrimination Task

Only cleaners with the D1 agonist SKF-38393 took less sessions to complete this task (SKF-38393 *vs.* Saline: $F_{1,9} = 5.49$, $p = 0.04$, Fig. 10) while no changes in their learning speed were

found with other compounds (SCH-23390 *vs.* Saline: $F_{1,9}$= 0.33, p= 0.58; Quinpirole *vs.* Saline: $F_{1,9}$ = 0.10, p = 0.76; Metoclopramide *vs.* Saline: $F_{1,9}$ = 1.82, p = 0.21, Fig. 10).

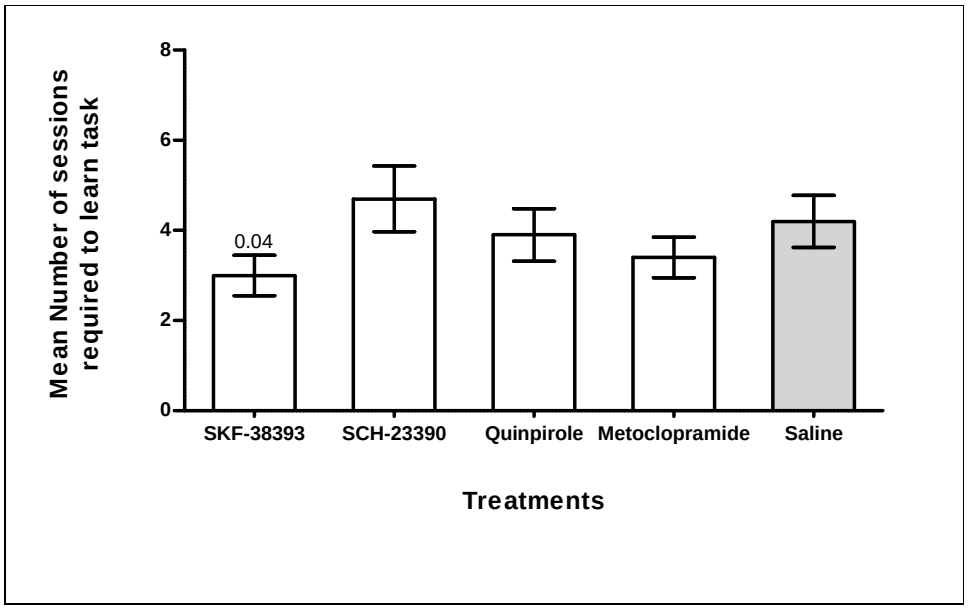

Figure 10 - The effect of SKF-38393, SCH-23390, Quinpirole and Metoclopramide on cleaner fish *L. dimidiatus* learning speed of a side discrimination task. Means are shown ± SEM. Probability value (shown above bar) refers to planned comparisons of least squares means effect of each dopaminergic treatment group against the reference (saline) group. Samples sizes were n = 10.

Discussion

The results presented in the previous section show that DA manipulation is indeed able to alter the learning speed of cleaner wrasses in both tasks (which varied in ecological relevance). Furthermore, these effects seem to be held exclusively via stimulation of the D1 pathway (D1 agonist -SKF-38393, Fig.9 and Fig.10), which lead to cleaner wrasses requiring less sessions to learn in both tasks. No other compound improved or delayed the learning speed of the cleaner wrasses tested in either task. However, quite interesting is that the same compound (D1 agonist) was able to produce significant effects in both tasks. This should be linked to the salience of reward (1 piece of shrimp) which is equal in both tasks. The perception of reward is thus key for the development of the learning process, without which behavioural reinforcement fails to occur and therefore the process cannot be completed (Schultz, 2006, 1998). These findings also suggest that the way cleaner wrasses respond to conditioned reward-predictive stimuli in the wild differs from the responses observed in these two experiments in laboratorial conditions.

Effects of D1 and D2 receptor blockade

Flagel and co-workers (Flagel et al., 2011b) showed that intact DA transmission was not always necessary to integrate CS-US association and to induce approach behaviour, as long as reward cues become effective predictors. Indeed, it was reported in a recent study (Darvas *et al.*, 2014) that animals were capable of associative learning with as little as 30% of DA base levels. Moreover, DA blockade seems to specifically impair associative learning in animals with a tendency to learn through a form of stimulus-reward learning where DA-mediated incentive salience is attributed to reward cues (Chow, 2011; Flagel et al., 2011b; Saunders and Robinson, 2012) but not of those who rely on the predictive value of such reward cues instead (Darvas *et al.*, 2014). In these experiments, none of the administered antagonists (D1 antagonist SCH-

23390, D2 antagonist Metoclopramide) delayed the learning ability of cleaner wrasses in either one of the tasks, when compared to control, probably because DA levels did not decrease to a degree where it would compromise the acquisition of a basal conditioned response. This is in accordance with previous studies that report that fully functional DA transmission is not necessarily required in learning tasks where reward cues develop into effective predictors (Darvas et al., 2014; Flagel et al., 2011b). DA is most likely not mediating the motivational properties of reward cues, but their predictive properties and continuous updating, which leads to cleaner wrasses learning both tasks at the same pace as the control group, even when their DA transmission is depleted.

Effects of D1 and D2 receptor stimulation

D1 receptor stimulation enhanced the learning speed in both tasks, which was surprising. It is known that increased DA transmission lead to improved cognitive functions and hence, improved learning (Eddins *et al.*, 2009) however, it was expected that a variation in ecological settings would result in a differential learning speed. Hence, a plausible hypothesis would be that increased DA levels generally improved cleaner wrasses' memory processes and facilitate the association of the reward cue but because the reward was similar in both tasks (1 piece of shrimp) lead to an enhancement of their learning ability irrespectively of experimental setting.

However, why would the same results seen with the D1 agonist failed to occur when the D2 agonist was administrated? Treatment with the D2 agonist Quinpirole has been reported to heavily impair animal judgment (St. Onge *et al.*, 2011) and locomotor activity (Mattingly *et al.*, 1993). It is also known that high levels of D2 receptor mRNA are found in animals that rely on the predictive properties of reward cues to learn (Flagel *et al.*, 2007). It may be possible that differences in receptor densities (putative lower densities) and the overall location of D2 receptors could have played an important role in the absence of results concerning the D2

agonist. In this case, the dosage used could also have an influence. Also, the predictive and repetitive structure of these experiments could also have an effect regarding the D2.

Concluding remarks

This study shows that DA signalling plays an important role in mediating the learning process of cleaner wrasses, mainly via D1 pathways, which works irrespectively of ecological relevance but in clear association with the value of reward. Interestingly, D1 and D2 blockade did not impair learning competence (compared to the saline treatment) since cleaner wrasses learned both tasks within 8 sessions. This potentially means that a CS-US association is achieved with presumably low DA levels, which is in accordance with previous studies (Darvas et al., 2014; Flagel et al., 2011b). Future work is clearly needed to further investigate the value of D1 pathways when distinct rewards are available. Also the probability of achieving a reward should be an important variable modulating the effect of DA agonists.

GENERAL DISCUSSION

These results show that DA influences cleaner wrasses behaviour mainly, but not exclusively, through D1 pathways. Furthermore, this influence tends to differ between newly acquired and previously acquired experience.

In the first experiment (Dopamine neuromodulation of cleaner wrasse cooperative behaviour, *in situ*), DA blockade rather than DA stimulation significantly altered cleaner wrasses behaviour. Due to a deficit in DA transmission, D1 blocked animals were unable to properly integrate the output of their own actions and therefore demonstrated difficulties in behavioural adjustment and a tendency to display risk-aversive behaviour. D2 blocked animals had similar results to D1; however they were not as severe, suggesting that DA transmission was not equally weakened with both antagonists (pathways). Neither D1 nor D2 stimulation (agonists) produced effects. This suggests that: a) D2 receptors might not be as important as D1 when mediating cooperative behaviour responses; b) DA transmission is essential for decision making when cooperating; c) higher levels of DA do not seem to influence cooperative behaviour when these perform previously learned behaviour (daily routines).

Conversely, in the second experiment (Dopamine neuromodulation of cleaner wrasse learning competence, *ex situ*), DA, in a context of learning a novel situation, stimulation rather than DA blockade significantly influenced cleaner wrasses' behaviour. D1 stimulated animals took significantly less sessions to learn the required task, contrarily to D2 stimulated and D1 and D2 blocked animals. This suggests that the pathways regarding D1 receptors should be crucial during the learning process of these animals, for example, in a context where fast associations are required.

To conclude, this study shows that intact DA transmission is crucial for the maintenance and performance of already established behaviours and associations, but not for the acquisition of

new ones. Future work will need to be done to clarify the exact involvement of DA receptors in these species' brain functions and other neuronal systems it might be working with. The present work provides important information regarding the physiological mechanisms involved in cleaning mutualisms and to what extent DA modulates them.

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